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QB355, V57  
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From: Li, Bao-Qun  
Sent: Friday, November 14, 2003 10:59 AM  
To: STIC-ILL

1. International Journal of Antimicrobial Agents 2002, Vol. 19(3), pp. 169-172.
  2. Virus Research 2002, Vol. 86(1-2), pp. 21-31.
- Thanks. CM1, 8E12.

AC 27

Vol 86

2002

pp. 21-31

11/14/03

# Enhanced protection against HSV lethal challenges in mice by immunization with a combined HSV-1 glycoprotein B:H:L gene DNAs

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Received 10 October 2001; received in revised form 5 February 2002; accepted 5 February 2002

## Abstract

The effectiveness of a cocktailed HSV-1 three-glycoprotein B, H, and L gene vaccine in comparison to individual glycoprotein gene vaccines was studied with regard to protecting against the HSV-1 infection. Three glycoprotein gene recombinant DNA vaccines, which produced the corresponding glycoproteins in Vero cells, were constructed using a CMV promoter. The cocktailed DNA vaccines were prepared by combining all three genes. The titers of neutralizing antibody following the immunization of the five vaccines were KOS(1/1024) > B:H:L = B(1/512) > H:L(1/64) > H(1/16) genes. The mice, which were immunized with L gene alone failed to induce enough neutralizing antibody. The CTL activity was rated as KOS (95%) > B:H:L (80%) > B(60%) > H:L(50%) > H (35%) gene vaccines at an E:T ratio of 50:1. The H gene alone or L gene vaccine alone induced little CTL activity. The protection rates of the DNA-vaccinated mice against the lethal intraperitoneal (i.p.) or i.m challenges were shown as KOS > B:H:L > B > H:L > H gene vaccines, and the protection activity depended on the lethal dosage of the challenging virus, which are inversely proportional to each other. Compared with the mice, which were vaccinated with individual DNA vaccines, the mice, which were vaccinated with the cocktailed three-gene vaccine, were shown to be better protected against the lethal challenging doses. It can be concluded that vaccination with the cocktailed three gene vaccines is more effective in protecting mice from the viral challenge and the protection rate varies inversely with the amount of lethal challenging dose used, although all DNA vaccines failed to block the latent infection in sensory nerves. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** HSV-1; DNA vaccine; Glycoproteins; Mice

## 1. Introduction

*Herpes simplex* virus type 1(HSV-1) is the causative agent of localized skin infections in the oral, ocular, and neural regions. The gly-

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proteins B, D, H, and L (gB, gD, gH, and gL) of HSV-1 are essential for infectivity in vitro, and they are the major viral proteins embedded in the virion envelopes along with cell membranes of the infected cells (Blacklaws and Nash, 1990; Browne et al., 1993; Ghiasi et al., 1995; Spear, 1993). The HSV-1 glycoproteins gB (Blacklaws and Nash, 1990; Ghiasi et al., 1996; Manservigi et al., 1990; Nesburn et al., 1994) and gD (Blacklaws and Nash, 1990; Ghiasi et al., 1995) have been focused on as subunit vaccines due to their primary roles in inducing cellular and humoral immune responses to the HSV-1 infection. In contrast, the role of HSV-1 gH:gL as a possible target of the immune system has been studied less extensively. gH and gL are presented as a heterodimer in the viral envelope and plasma membranes of HSV-1 infected cells (Huchinson et al., 1992). It is known that monoclonal antibodies (MAbs) to the gH:gL complex can inhibit HSV-1 infections in vitro (Buckmaster et al., 1984). Furthermore, immunization in mice with recombinant forms of gH (Ghiasi et al., 1992a, 1994a), gL (Ghiasi et al., 1994b) or gH:gL (Browne et al., 1993) induced immune responses, which can actually protect mice from a lethal HSV-1 challenge when administered passively. Previously we produced the HSV-1 gB (Cha et al., 2000) and gH (Kang et al., 2000) proteins using a baculovirus expression system (Lee et al., 1998a,b), and it successfully formed neutralizing antibodies in mice. A novel approach for vaccination is the induction of immune responses against an antigenic protein, which was expressed in vivo from an introduced gene. In animal models, such DNA immunization has been recently shown to induce both humoral and cellular immune responses against a range of pathogens and it is also highly effective in inducing long-lived memory responses (Tang et al., 1992; Fynan et al., 1993). DNA immunization using HSV-1 gB gene against HSV-1 infection was reported by Manickan et al. (1995), Hariharan et al. (1998), where they found protective activity in mice. In addition, there is a possibility that a mixture of several glycoproteins with similar functions would provide better protection than individual glycoproteins against infections (Ghiasi et al., 1996).

Here, three glycoproteins (gB, gH, and gL) were selected, which play roles in the attachment of the virion to the cell and penetration of the virion into cells (Cai et al., 1988; Navarro et al., 1992; Huchinson et al., 1992). A comparison was made of the protection efficacy of a mixture of three HSV-1 gB, gH, and gL genes with three individual gene vaccines against various lethal challenges. This current study is designed to investigate the followings: the production of glycoprotein in vero cells, the ability of individual gene vaccines to be protected from lethal intraperitoneal (i.p.) and intradermal (i.m.) challenges with HSV-1, the induction of cellular and humoral immune responses, and the protection against the establishment of HSV-1 latency.

## 2. Materials and methods

### 2.1. Virus, cells and media

*H. simplex* virus type 1 (HSV-1) strain F (ATCC VR-733) (Rockville, MD, USA) and HSV-1 strain KOS were obtained from the Korean National Institute of Health, (Seoul, Korea). KOS strain was used as a positive control vaccine. HSV-1 strain McKrae (a neurovirulent strain) (B.T. Rouse, University of Tennessee, USA) was used as a challenge virus. The vero cell line (ATCC CCL 81) was obtained from the Korean Type Culture Collection (KTCC, Seoul, Korea). The vero cells grew as a monolayer in a plastic tissue culture flask of an Eagle's minimum essential medium (MEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 0.2% sodium bicarbonate (Sigma, St Louis, MO, USA) and 50 µg/ml gentamycin (Gibco) at 37 °C, subcultured every 3 days. EL4 cell (H-2b) (lymphoma) (ATCC TIB-40) was obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI 1640 (Gibco) supplemented with 10% FBS, 7 mm of L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), and 0.05 mm of 2-mercaptoethanol.

### 2.2. Bacterial strains, plasmids and animals

The pcDNA3.1(+) in *Escherichia coli* XL1-Blue (Invitrogen, San Diego, CA, USA) and the

GEM-T plasmid (Promega Biotech, Madison, WI, USA) were used for cloning of the HSV-1 genes. The pGus-gB3.1 plasmid (Cha et al., 2000) and the pHcGH plasmid (Kang et al., 2000) in *E. coli* XL1-Blue were used for HSV-1 gB and gH gene sources, respectively, and they were maintained in a LB broth (Gibco). Six to 8-week-old female Balb/c (H-2b) and C57BL/6 (H-2d) mice were obtained from Daehan Biolink Co (Eum-sung, Chungbuk, Korea).

### 2.3. Virus purification and titration

Approximately  $2 \times 10^7$  vero cells were seeded in a 75 cm<sup>2</sup> tissue culture flask and incubated for 72 h at 37 °C for attachment and growth. The cells were infected with the HSV-1 strains F, KOS and/or McKrae at an m.o.i. of 1.0 pfu per cell. After 1 h of adsorption at 37 °C, the monolayers were washed with phosphate-buffered saline (PBS) (pH 7.4), and the cells were incubated with 15 ml of the medium for 35 days at 37 °C. After a complete cytopathic effect, cells and supernatants were harvested (Dubin and Jang, 1995). Titers in the virus solution were determined by a plaque assay (Lee and Miller, 1978) with a slight modification; the method utilizes 0.8% agarose (Sigma) in a supplemented MEM medium as an overlay, and vero cells were cultured and infected at 37 °C.

### 2.4. Oligonucleotides and antibody

The primers (L-1, 5':5'-TGCAGATCTGCGC-TATGGGGATTTTGGGT-3'; and L-2, 3':5'-TGCAGATCTGTTTCCGTCGAGGCATCGT-3') were deduced from the terminal region of the published sequence of the gL gene of HSV-1 strain F (Dubin and Jang, 1995) and used for PCR of the HSV-1 gL gene. The primers contained a *Bgl*III site (underlined) at both ends. The primers B-1, 5':5'-TGCAGATCTGCGCTATGGGATTTTGGGT-3'; and B-2, 3':5'-TGCA-GATCTGTTTCCGTCGAGGCATGGT-3') were used to detect latent HSV-1 gL gene in ganglia (Ramakrishnan et al., 1994). The oligonucleotides were synthesized by Korea Biotech Inc., Daejeon, Korea. The HSV-1 gB-specific monoclonal anti-

body was purchased from Biodesign International (Main, USA). The HSV-1 gH specific monoclonal antibody 52S was obtained from Dr U. Gompel, University of London, UK. The gL-specific monoclonal antibody was obtained from Dr Long, Northwestern University (Chicago, USA).

### 2.5. Restriction enzyme digestions and agarose gel electrophoresis

All restriction endonuclease digestions were performed according to the manufacturer's instructions. The DNA genome and vector DNAs were digested and electrophoresed on 1.0% agarose gels. The molecular size of each DNA fragment was determined by comparing their mobility rate with 123 bp DNA ladders. The reactions were terminated by adding 1/10 volume of the stop solution. Details of the gel electrophoresis and visualization of the DNA fragments were previously described by Lee and Miller (1978), Sambrook et al. (1989).

### 2.6. Purification of plasmids

The plasmids DNAs in *E. coli* were purified using Qiagen DNA purification columns following the manufacturer's instructions (Qiagen, MD, USA). For the DNA vaccine preparation, after being washed in 70% ethanol, the DNA was redissolved in a sterile phosphate buffered saline (PBS) to obtain a final concentration of 1 µg/µl, aliquoted and stored at -20 °C until it was injected.

### 2.7. Construction of DNA vaccines with HSV-1 gB, gH, or gL genes

Three gB, gH, and gL gene sequences were cloned into the plasmid pcDNA3.1 (Invitrogen). Cloning was carried out by mixing together 15 µl (0.2 µg) of the insert DNA, 20 µl (0.1 µg) of vector DNA, 5 µl of 5 mM ATP, 5 µl of 10 × T4 DNA ligase buffer, 2 µl (1.8 U/µl) of T4 DNA ligase, and 3 µl of distilled water. Then, the total 50 µl mixture was reacted at 14 °C for 18 h. The reaction condition was examined a 1.0% agarose

gel electrophoresis. DNA from the low melting agarose gel was eluted using an electroelutor. Competent *E. coli* cells were prepared and transformed with the recombinant plasmids according to the procedure as described by Sambrook et al. (1989).

The gB gene of HSV-1 in the plasmid pGus-gB3.1 (Cha et al., 2000) was digested out with 1 unit of *Kpn*I and cloned into the *Kpn*I site of the pcDNA3 vector using the procedure that was described above. The clones were screened, identified by Southern blotting (Southern, 1975), partially sequenced (Sanger et al., 1977; Uh et al., 2001a,b), and finally named pHS1-gB1 (8.5 kb) recombinant vaccine plasmid.

The HSV-1 gH gene in the pHcGH plasmid (Kang et al., 2000) was digested with 2 units of *Bgl*II to generate 2.5 kb DNA. Then, the 2.5 kb DNA fragment was cloned into the *Bam*HI site of the pcDNA3.1 vector and confirmed by a partial sequencing of the cloning site. The recombinant plasmid was isolated and was named pHS1-gH (7.9 kb) vaccine plasmid.

The gL gene of the HSV-1 strain F was amplified with a PCR (Ramakrishnan et al., 1994) using two primers containing *Bgl*II sites. The resulting 718 bp of the gL gene was inserted into the *Bgl*II site of pGEM-T plasmid, and then it was transferred into the *Bam*HI site of pcDNA3.1 vector using the procedure described above. This was named pHS1-gL (6.1 kb) recombinant plasmid vaccine. The insertion of the gL gene was confirmed by the partial sequencing process.

## 2.8. Immunoprecipitation and Western blot analysis

In order to detect glycoprotein productions in cells, a radioimmunoprecipitation analysis was carried out using the procedure as described by Cha et al. (2000). Western blot analysis was used for detecting the glycoproteins using the procedure described by Sambrook et al. (1989).

## 2.9. DNA vaccine immunization of mice

DNA vaccination to mice was carried out using a modification of the procedures described by Lee

et al. (1999). The glycoprotein gene vaccines (pHS1-gB, pHS1-gH, pHS1-gL, pHS1-gH:gL complex, and pHS1-gB:gH:gL cocktail), which were constructed and formulated for this work, were injected directly into regenerating quadriceps muscles of the five antigenic groups (ten mice per group) of female C57Bl/6J mice weighing 19–21 g. At 0, 7, and 14 days, 100  $\mu$ l (1.0  $\mu$ g/1  $\mu$ l) of the DNA solutions were intramuscularly injected into quadriceps muscles undergoing regeneration using 28.5-gauge syringes. To promote vaccination, 100  $\mu$ l of 10 mM (10 mM in PBS) cardiotoxin derived from snake-venom (Latoxan, Rosans, France) was injected into the muscles 5 days before vaccination. Mice immunized with HSV-1 strain KOS (TCID<sub>50</sub>;  $2 \times 10^6$  pfu) served as the positive control, and PBS served as the negative control vaccine.

The pHS1-gB:gH:gL and pHS1-gH:gL vaccines contained the same total amount of HSV-1 glycoprotein gene DNAs which were used in pHS1-gB alone. Each of the glycoprotein genes in the pHS1-gB:gH:gL vaccine was present in one-third of the concentration of pHS1-gB in the pHS1-gB vaccine. The pHS1-gB vaccine contained 100  $\mu$ g of pHS1-gB DNA per inoculation. The cocktail vaccine contained 100  $\mu$ g each of the three-glycoproteins gene per inoculation.

## 2.10. Neutralization assay

Virus neutralization assay was performed using the procedures described by Cha et al. (2000), Kang et al. (2000) with a slight modification. The mice, which were immunized with the five DNA vaccines and two control groups, were left to bleed at 14 days after the second vaccination. Blood from each group was heated at 56 °C for 30 min to inactivate the complement. One hundred microlitre of the heat-inactivated serum was added into a 96-well, flat-bottom microtiter plate for serial dilution (Falcon, Lincoln Park, NJ, USA). One hundred microlitre of  $200 \times$  TCID<sub>50</sub> of the live KOS strain was added to the plate, sealed and incubated for 18 h at 4 °C. Then 100  $\mu$ l of  $5 \times 10^3$  viable vero cells were added to each well and incubated at 37 °C for 5 days. The serum dilution factor that neutralized 50% of the

virus was determined as the titer. The TCID<sub>50</sub> was 0.69 pfu as determined by the procedure described by Reed and Munch (1938).

### 2.11. Cytotoxic T-lymphocyte assay

Cytotoxic T-lymphocyte assay was performed using a CytoTox96-nonradioactive cytotoxicity assay kit (Promega Biotech) as described by Korzeniewski and Callewaert (1983). The spleens of the immunized mice with the five DNA vaccines and two control groups were perfused with a saline solution 2 weeks after the final DNA immunizations were carried out and settled for 15 min. Then the supernatant of each group was pooled and washed at  $1000 \times g$ . Pelleted cells were loaded on the top of the 4 ml of Ficoll-Paque solution (Pharmacia) and centrifuged at  $1000 \times g$  for 40 min to remove debris. The cell layer in the Ficoll-Paque gradients was collected, washed, and resuspended in an MEM medium (Gibco) containing 10% FBS. The spleen cells were cultured overnight in a MEM medium containing recombinant interleukin-2 (IL-2) (Promega Biotech) at 10 IU/ml. The IL-2-stimulated spleen cells were harvested after a 1 day culture and washed twice. For preparation of the target cells, EL-4 cells were infected with HSV-1 at m.o.i. of ten and then incubated for 4 h. The IL-2-stimulated spleen cells (effector cell) were diluted to make up concentrations of  $100 \times 10^3$ ,  $50 \times 10^3$ ,  $20 \times 10^3$ , and  $10 \times 10^3$  viable cells per 100  $\mu$ l, mixed and cultured with a constant number of  $2 \times 10^3$  target cells in 100  $\mu$ l of MEM medium in round-bottomed wells. After a 4 h incubation process at 37 °C, 100  $\mu$ l of the supernatant was collected for the lactate dehydrogenase (LDH)-release assay (Promega Biotech). Cytotoxicity (%) was calculated using the Promega formula (Promega Biotech).

### 2.12. Intraperitoneal virus challenge test

The mice, which were immunized with the five DNA vaccines, positive and negative control vaccines were challenged with the McKrae strain using a modified procedure as described by Cha et al. (2000), Ghiasi et al. (1996). Two weeks after the final vaccination (100  $\mu$ g DNAs per 100  $\mu$ l per

mouse), the mice were challenged with  $5 \times 10^6$  ( $1.5 \times \text{LD}_{50}$ ),  $5 \times 10^5$ ,  $5 \times 10^4$ ,  $5 \times 10^3$  pfu of lethal i.p. doses of the McKrae strain. The challenged mice were monitored for 2 weeks and the effects were scored as morbidity and mortality. The lethal challenge dosage of McKrae strain represents the minimal amount of the virus, which kills 100% of the PBS-infected negative control as previously described by Ghiasi et al. (1996).

### 2.13. Intradermal virus challenge test

The left flanks of the mice immunized with the five DNA vaccines and two positive and negative control vaccines (eight Balb/c mice per group) were challenged with the McKrae strain at 2 weeks after the final vaccinations took place as described by Simmons and Nash (1984) and Lee et al. (1999). After depilation and anesthetization, 2  $\mu$ l of  $2 \times 10^7$  (40  $\text{LD}_{50}$ ) pfu of the McKrae strain per mouse was inoculated at the scarifications, then the challenged mice were monitored for 2 weeks. The signs of skin lesions and mortality were observed twice a day. The degree of the symptoms was scored according to five criteria as previously described (Simmons and Nash, 1984; Lee et al., 1999).

### 2.14. Detection of latent viral DNAs in ganglia using PCR

The immunized mice were inoculated in the left flank with approximately 2  $\mu$ l of  $5 \times 10^6$  pfu of the McKrae strain following scarification at 2 weeks after the final vaccinations took place. The strain McKrae DNAs in the infected ganglia were then purified and amplified with a PCR analysis as described by Ramakrishnan et al. (1994) using B-1 and B-2 primers.

## 3. Results and discussion

### 3.1. Humoral responses induced by the glycoprotein gene vaccines

Recombinant DNA vaccines expressing HSV-1 F strain gB, gH, and gL proteins, were con-

structed using the conventional CMV promoter-based pcDNA3.1(+) vector as described in Section 2. The productions of HSV-1 gB, gH and gL proteins in Vero cells which were transfected with the three gene vaccines (pHS1-gB, pHS1-gH, and/or pHS1-gL) were detected by applying a radioimmunoprecipitation analysis (data not shown). These results support earlier conclusions that HSV-1 glycoproteins were produced in mammalian cells (Gompels and Minson, 1989; Hutchinson et al., 1992; Peng et al., 1998) and insect cells (Ghiasi et al., 1992a,a,b; Cha et al., 2000; Kang et al., 2000; Westra et al., 1997 using recombinant viruses.

The protection rates of a mixture vaccine of three HSV-1 genes (gB, gH, and gL) and a two gene complex (gH:gL) vaccine with three individual gene vaccines against various lethal challenges were compared. The primary goal was to determine whether these mixtures would produce better protection than the individual glycoprotein gene vaccines. The expression of the cocktail gene vaccine pHS1-gB:gH:gL and pHS1-gH:gL complex vaccine was compared with that of the individual pHS1-gB, pHS1-gH, and pHS1-gL vaccines. The cocktail vaccine contained the same total amount of DNAs. Mice were vaccinated with an equal amount of each of these gene vaccines. HSV-1 strain KOS and PBS were used as a positive and negative control, respectively.

Sera that were collected from the mice which were injected with the five different DNA vaccines were compared with sera from the mice injected with positive (KOS) and negative PBS controls with respect to their neutralizing activity (Fig. 1). The live KOS vaccine serum had a neutralizing titer of 1/1024. The sera, which were immunized with the pHS1-gB and the pHS1-gB:gH:gL vaccines, contained equal neutralization titers of 1/512. The neutralizing titers of the sera from mice, which were immunized with the pHS1-gH:gL, pHS1-gH, and gHS1-gL vaccines, were 1/64, 1/16, and 1/4, respectively. The mice sera from the PBS control and pHS1-gL vaccines showed little or no neutralizing activity. These results indicate that the DNA vaccines can induce a humoral immune response in mice, which is shown as  $\text{pHS1-gB:gH:gL} = \text{pHS1-gB} > \text{pHS1-gH:gL} > \text{pHS1-gH}$  vaccine. In contrast, Manickan et al. (1995) reported that low levels of neutralizing antibody titers of 1/16 and 1/32 from the mice sera, which were immunized with gB gene and the KOS, strain, respectively. Furthermore, there are several reports made where the individual glycoprotein (gB or gH) vaccines originated from insect cells (Ghiasi et al., 1996; Cha et al., 2000; Kang et al., 2000) and (gH, gL and gH:gL) vaccines obtained from mammalian cells (Peng et al., 1998; Browne et al., 1993) induced high titers of HSV-1 neutralizing antibodies in animal models. The high neu-

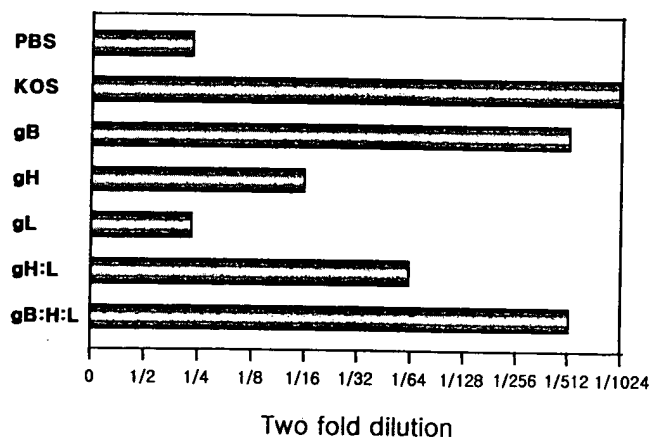


Fig. 1. Neutralizing antibody titers in the mice vaccinated with different DNA vaccines. Mice were vaccinated one or three times with DNA vaccines. Sera were collected 3 weeks later from ten mice per group and HSV-1 neutralizing antibody titers were determined for each serum as described in Section 2.

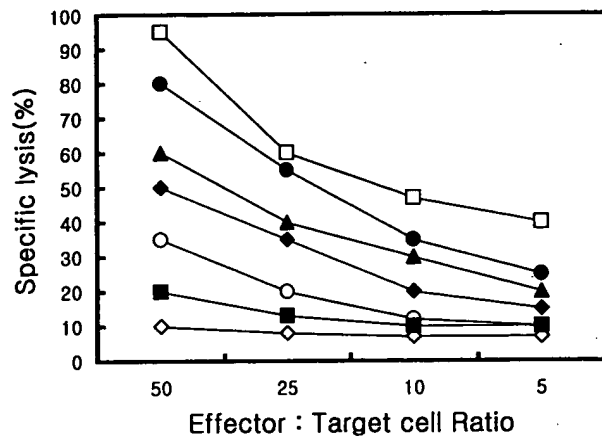


Fig. 2. Comparison of cytotoxic activity in mice vaccinated with different DNA vaccines. Mice were immunized twice with 50  $\mu$ g of DNA vaccines and then challenged with DNA vaccines. The specific cytotoxic activities were measured in spleen cells from mice after nonspecific stimulation took place with IL-2 and ConA for 2 days ex vivo as described in Section 2. Vaccine symbols: live (KOS) (□), pHS1-gB:gH:gL (●), pHS1-gB (▲), pHS1-gH:gL (◆), pHS1-gH (○), pHS1-gL (■), and mock (◇).

tralizing titer in serum immunized with the KOS strain may be due to the presence of more potential protective epitopes in the live virus (Ghiassi et al., 1996). Since the cocktail DNA vaccine and the pHS1-gB vaccine showed a similar neutralizing activity (1/512), other subunit vaccines in the cocktail vaccines (pHS1-gH and/or pHS1-gL subunit-vaccines) do not seem to play a major role in inducing neutralizing antibodies.

### 3.2. Cytotoxic T lymphocyte response

CTL activity was detected in the spleen cells of the mice infected with the KOS strain, the mice injected with the five different DNA vaccines, and the mice injected with mock vaccine in vitro (Fig. 2). The mice immunized with KOS, pHS1-gB:gH:gL, pHS1-gB and pHS1-gH:gL, induced high levels of CTL activities (95, 80, 60, and 50%, respectively) at E:T ratio of 50:1 infected with the McKrae strain. At the E:T ratios of 25:1, 10:1 and 5:1, the specific lysis value decreased in a similar pattern to that of the 50:1 ratio. The mice immu-

nized with pHS1-gH alone induced a modest level of CTL activity and the mice immunized with pHS1-gL induced little or no detectable CTL activity at all ET ratios as shown in the case of the negative control. According to these results, they showed that the mice immunized with the gB:gH:gL cocktail, gB alone, and gH:gL complex DNA vaccines induced higher levels of CTL activity than those of the gH or the gL vaccine alone. The rank order for the CTL activity is shown as KOS > pHS1-gB:gH:gL > pHS1-gB > pHS1-gH:gL > pHS1-gH vaccine. The cocktail vaccines showed higher CTL responses than individual vaccines most likely due to the synergic roles of the three subunit-gene DNAs in the cocktail. Similarly, Manickan et al. (1995) reported that the mice immunized with gB gene showed a low level of CTL activity than those with the KOS strain. Furthermore, Hariharan et al., (1998) observed 65–75% specific cell lysis of the CTL activities at E:T ratio of 30:1 using 0.01–100  $\mu$ g DNA vaccines of the gB gene of HSV-1 strain KOS. The dosage of our DNA vaccines was 100  $\mu$ g per mouse, and the value of the CTL activity was similar to the results derived from Hariharan et al. (1998) in gB gene vaccine alone, on the other hand it was higher in the cocktail vaccine. However, it was found that 1/1000  $\mu$ g could actually induce a similar immune response.

### 3.3. Protections from the dose dependent HSV-1 lethal i.p. challenge

Protection activity was detected in the mice, which were immunized with DNA vaccines. The mice were i.p. challenged with high to low lethal dosages ( $5 \times 10^6$ ,  $5 \times 10^5$ ,  $5 \times 10^4$ ,  $5 \times 10^3$  pfu per inoculum) of the McKrae strain (Fig. 3). The entire KOS-immunized groups were completely (100%) protected from death at all lethal doses, while the negative vaccination had no effect. At the  $5 \times 10^6$  pfu of lethal dose challenge, the pHS1-gB:gH:gL cocktail vaccine and the pHS1-gB vaccine protected mice as much as 90 and 80% of the mice from death from 7 to 14 days postinfection (pi), respectively. The pHS1-gH:gL vaccine protected 50% of the mice from 7 to 14 days pi, but



pHS1-gH, pHS1-gL and mock vaccinations failed to protect the mice from death. The protective rate of the DNA vaccines immediately increased when the challenging virus doses were reduced.

At the  $5 \times 10^5$  pfu of lethal challenge, the pHS1-gB:gH:gL and pHS1-gB vaccines like the KOS strain showed 100% protection of the mice from death for 14 days pi. The pHS1-gH:L complex vaccine protected up to 100% of the mice within 5 days pi, and 90% of the mice from 6 to 14 days pi. The pHS1-gH vaccine protected 100% of the mice within 4 days pi, 80% from 5 to 6 days pi, and 50–40% from 7 to 14 days. The pHS1-gL vaccine alone also protected up to 60% from 5 to 6 days, after 6 days pi the protection rates immediately decreased to 30%. The mock vaccine failed to protect the mice from death.

At the  $5 \times 10^4$  pfu and the  $5 \times 10^3$  pfu of lethal dose challenges, the pHS1-gB:gH:gL, pHS1-gB,

and pHS1-gH:gL vaccines protected up to 100% of the mice from death for 14 days pi. The pHS1-gH vaccines protected up to 90% from 6 to 7 days, and after 7 days pi, the protection rate decreased 60%, and the pHS1-gL vaccine also increased the protection of up to 40% of the mice from death from 8 days at  $5 \times 10^4$  pfu. However, at  $5 \times 10^3$  pfu of the lethal dose challenge, pHS1-gH vaccine increased the protection of up to 90% activity of the mice from 8 days pi, and pHS1-gL vaccine protected 60% of the mice from death. The mock infection of the mice also protected 50% of the mice from death. This means that the natural protection of the mice themselves is provided.

These results demonstrate that the protection activity depends on the lethal dosage of the challenging virus and they are inversely proportional to each other. The protection rate of the vaccines is shown as  $\text{KOS} > \text{pHS1-gB:gH:gL} > \text{pHS1-}$

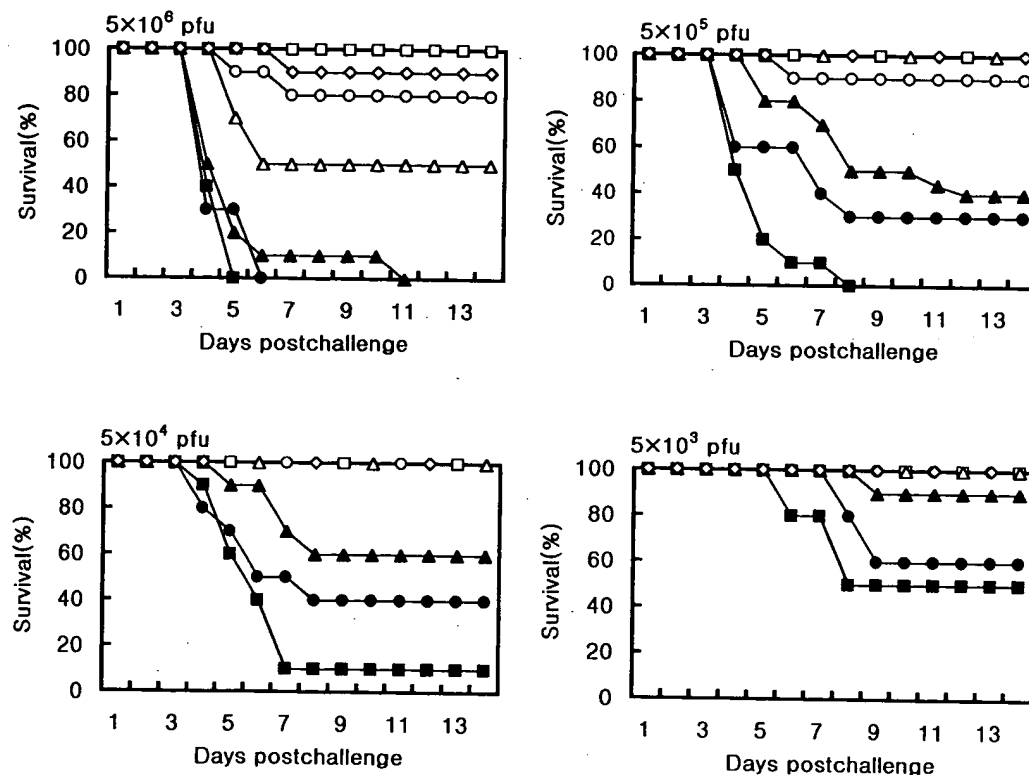


Fig. 3. Survivals of vaccinated mice following lethal i.p. challenge with HSV-1. Mice were vaccinated three times and then challenged i.p. with lethal doses  $5 \times 10^6$ ,  $5 \times 10^5$ ,  $5 \times 10^4$ ,  $5 \times 10^3$  pfu of the McKrae strain as described in Section 2. Vaccine symbols: live (KOS) (□), pHS1-gB:gH:gL (◇), pHS1-gB (○), pHS1-gH:gL (△), pHS1-gH (▲), pHS1-gL (●), and mock (■).

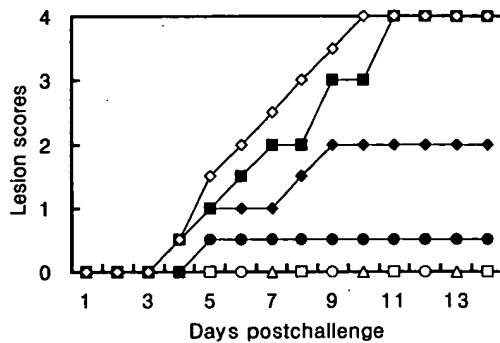


Fig. 4. Survivals and zosteriform scores of the vaccinated mice following lethal i.m. challenges with HSV-1. Mice were vaccinated three times and then i.m. challenged with  $2 \times 10^7$  pfu per mouse of the McKrae strain as described in Section 2. The severity occurring on the mice was scored as follows; phase 1, just perceptible small vesicle; phase 2, occurrence of erosion and ulceration of local lesions; phase 3, occurrence of ulceration; phase 4, occurrence of severe ulceration; and phase 5, death. Vaccine symbols: live (KOS) (□), pHS1-gB:gH:gL (○), pHS1-gB (△), pHS1-gH:L (●), pHS1-gH (◆), pHS1-gL (■), and mock vaccine (◇).

gB > pHS1-gH:gL > pHS1-gH > pHS1-gL vaccines. It is assumed that the expression of the cocktail DNA vaccine in the mammalian vectors results in the synthesis of authentic glycoproteins and consequently induces cooperative protection against lethal challenges.

#### 3.4. Protection from HSV-1 lethal i.m. challenge

Using the zosteriform model to assess immunity (Simmons and Nash, 1984; Lee et al., 1999), the protection of the vaccinated mice was observed after a lethal i.m. challenge for 14 days with  $2 \times 10^7$  pfu per mouse of HSV-1 strain McKrae, and the progress of the resulting zosteriform lesions (day 1–14) is illustrated in Fig. 4. Most of the skin lesions appeared on the fourth to fifth day following inoculation. Initial lesions were punctuated, and slightly depressed erosions were observed in the area of the dermatome involved. The lesions were enlarged within 2 or 3 days and coalesced to form a unilateral band-like lesion, which were subsequently ulcerated (data not shown). These observations are similar to the reports made by Simmons and Nash (1984), Lee et al. (1999). The positive KOS-vaccinated mice

were completely (100%) healed and protected from death for 14 days pi, while the negative PBS- and pHS1-gL-vaccinated mice showed the severity phase-1 from 4 day pi and finally died after 10 days pi with lesions. This result means that the negative and gL gene failed to protect the mice from death. The pHS1-gB:H:L cocktail vaccine and pHS1-gB vaccine also completely (100%) protected the immunized mice as much as the positive KOS-control group from death for 14 days pi. This protection is similar to the case of the i.p. injection observed above. The pHS1-gH:gL complex vaccine protected the immunized mice in the phase 1 stage of the progression of lesions on the mice. The rank order of protection rates is shown as KOS = pHS1-gB:gH:gL = pHS1-gB > pHS1-gH:gL > pHS1-gH vaccines. This result again suggests that the cocktail DNA vaccine expressed antigenic glycoproteins in the mice, which work cooperatively for providing protection against lethal i.m. challenge dosage. In fact, similar results were reported by Manickan et al. (1995), where it was found that 80% of the gB DNA-immunized Balb/c mice survived after being challenged with 10 LD<sub>50</sub> of HSV-1. Hariharan et al. (1998) have also found that 100 and 30 µg of the gB gene vaccine of strain KOS were able to protect completely from the appearance of zosteriform lesions at lethal challenge of the McKrae strain. It could be concluded that the cocktail and gB gene DNA vaccines seem to be equally effective in i.m. challenges.

#### 3.5. Effect of vaccination on establishment of latent infection

To test whether the DNA vaccinations would actually prevent the development of a latent HSV-1 infection, the vaccinated mice were post immunized for 2 weeks with a  $5 \times 10^6$  pfu of the lethal dose challenge of the HSV-1 strain McKrae on the left flank route. All of the ganglia from the mice, which were immunized with DNA vaccines and control groups, were not protected from developing latent infections (Fig. 5). Ghiasi et al. (1996) found that no latency was detected in the mice, which were vaccinated with a seven-glycoprotein mixture. These results strongly suggest

that a cocktail of three glycoprotein genes is likely to be a better subunit vaccine than any individual glycoprotein gene vaccine available. In order to determine which of the eight currently known herpes glycoprotein genes should be included in a cocktail, and what ratio of glycoprotein genes in a cocktail would produce the best efficacy will take some time.

### Acknowledgements

This work was supported by grant No. KOSEF 96-0401-11-01-3 which was received from the Korea Science and Engineering Foundation.

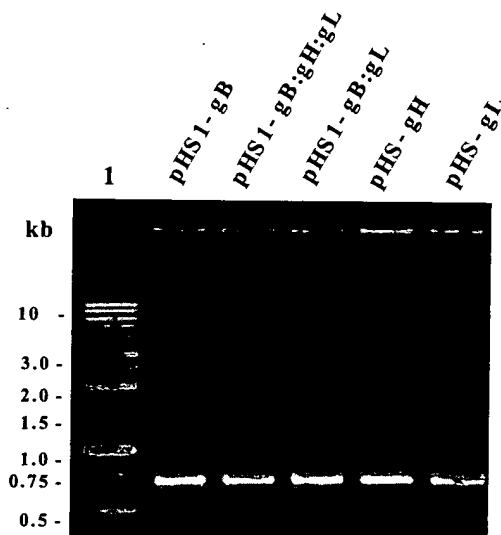


Fig. 5. Detection of HSV-1 genomes by a PCR using gB gene primers from ganglia in immunized mice after lethal challenge. Mice were vaccinated three times with different DNA vaccines into the quadriceps muscle followed by infection of the HSV-1 strain McKrae. The ganglion was removed 4 weeks after the final injection and immediately frozen in liquid N<sub>2</sub>. Then the HSV-1 genomes in the ganglia were amplified by a PCR using gB gene primers and the PCR products were run on a 1.2% agarose gel. Lane 1, 1 kb DNA ladder, lanes 2–6 used for the ganglionic DNA extracts of mice vaccinated with pHS1-gB:gH:gL, pHS1-gB, pHS1-gH:gL, pHS1-gH, pHS1-gL vaccines, respectively.

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